

## **REMARKS**

Claims 1-7, 9, 30-34, 37, 39-43, 45, 49, 55, 62, and 98 were amended. Claims 58-60, 68-71, and 81-97 are cancelled. New claims 99-141 were added. These new claims were added to correct the multiple dependencies objected to by the Examiner and accordingly no new matter has been added. Claims 1-57, 61-67, 72-80, 98-141 are currently pending.

The specification has been amended to correct typographical errors and to include SEQ ID NO identifiers. A new sequence listing (a hard copy) and diskette, along with a verified statement are also being filed herewith to incorporate SEQ ID NOs for sequences that were present in the originally filed specification.

### **Claim objections**

The claims have been amended to correct the objections raised by the Examiner (multiple dependent claims depending upon other multiple dependent claims and lack of sequence identifiers). Accordingly, applicant respectfully requests withdrawal of these claim objections.

### **Claim rejections under 35 U.S.C. §112, second paragraph**

The Examiner has rejected certain claims as being indefinite. Applicants submit the present claim amendments have rendered this rejection moot and accordingly request withdrawal of this ground of rejection. Specifically the claim amendments clarify more particularly the recitation of “P,” “A,” and “X” in the claims.

### **Claim rejections under 35 U.S.C. §112, first paragraph (enablement) and written description**

The Examiner has rejected claims 32-34 (the dimer claims) under 35 U.S.C. 112, first paragraph for lack of enablement and written description. Claims 32-34 have been amended to depend from claims further describing the dimer (which include language describing the epitope to which the antigen binding fragment is capable of binding or cross-reacting). Accordingly, applicants submit that the present claim amendments render this ground of rejection moot and accordingly request withdrawal of this ground of rejection.

### **Claim rejections under 35 U.S.C. §102**

Certain claims have been rejected under 35 U.S.C. §102(b) as being anticipated by Ward et al. The present claims have been amended to recite “human” antibody multimers. Ward et al. do not teach or suggest a human antibody, much less teach or suggest how one would make the claimed human multimer that is capable of reacting with the claimed epitopes of Formula I, II, or III. Rather Ward et al. only teach a mouse monoclonal antibody.

Certain claims have been rejected under 35 U.S.C. §102(b) as being anticipated by Snapp et al. The present claims have been amended to recite “human” antibody. Snapp et al. do not teach or suggest a human antibody, much less teach or suggest how one would make the claimed human multimer that is capable of reacting with the epitopes of Formula I, II, or III. The Examiner notes that Snapp et al. disclose “the monoclonal antibody KPL-1 which binds to amino acid residues 5-11 (YEYLDYD) of PSGL-1, wherein at least one tyrosine is sulfated.” However, this conclusion is retracted in a latter article (Thatte, et al., J. Leukoc. Biol. 72: 470-477; 2002), whose authors include Snapp. Thatte et al. conclude that the mouse monoclonal antibody KPL1 does not require sulfatation for binding to its target: “KPL1 recognition of PSGL-1 does not require tyrosine sulfation.” (See page 474, 2nd column), and further in the discussion: “This suggests that recognition of PSGL-1 by either of [PL1 and KPL1] is sulfation-independent.” (See page 475, 1<sup>st</sup> column). A copy of this article is included herewith. In contrast to the teachings of Thattee et al. (regarding KPL-1), the human multimers of the present invention (and as claimed) require sulfation of the target epitope for binding.

Accordingly, applicants respectfully submit that Ward et al. and Snapp et al. do not teach nor suggest each and every claimed element, and request withdrawal of these ground of rejections.

**Provisional double patenting rejection over claim 29 of co-pending Application No:  
10/029,988**

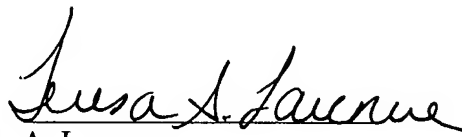
The Examiner has provisionally rejected claims 1-28 and 32-48 over claims 18-21 and 23-117 of co-pending Application No: 10/032,423. Applicants respectfully submit that since no claims have been allowed, this rejection is not timely and should be withdrawn. Once the co-pending application issues, if necessary at that time, a terminal disclaimer will be filed in the appropriate application.

**CONCLUSION**

Applicant respectfully requests entry of the present claim amendments and response and consideration of the above-identified application on the merits. It is believed that the application is in condition for allowance and such action is earnestly requested. If the Examiner wishes to discuss the present application, the Examiner is respectfully invited to contact the undersigned. The Office is authorized to charge any fees or credit any refunds due to Kenyon & Kenyon's Deposit Account No: 11-0600.

Respectfully submitted,  
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# Binding of function-blocking mAbs to mouse and human P-selectin glycoprotein ligand-1 peptides with and without tyrosine sulfation

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**Abstract:** P-selectin glycoprotein ligand-1 (PSGL-1) mediates rolling of leukocytes on P-selectin-expressing endothelial cells under shear flow. Function-blocking monoclonal antibodies (mAbs) against mouse and human PSGL-1 recognize an anionic segment at the N-terminus of PSGL-1. High affinity interaction of PSGL-1 with P-selectin requires sulfation of tyrosines 46, 48, and 51 (human) or 54 and 56 (mouse). We tested binding of two anti-human (KPL1 and PL1) and two anti-mouse (4RA10 and 2PH1) PSGL-1 mAbs to synthetic peptides of N-terminus of human and mouse PSGL-1 and found binding to be independent of tyrosine sulfation. In peptide-blocking experiments, sulfated and nonsulfated human and mouse peptides competed with antibody binding to PSGL-1 expressed on myeloid cells. Arylsulfatase treatment significantly reduced P-selectin binding but had no effect on antibody binding. Our data show, in three independent assay systems, that function-blocking antibodies to mouse or human PSGL-1 do not require sulfation of N-terminal tyrosines for binding. *J. Leukoc. Biol.* 72: 470–477; 2002.

**Key Words:** arylsulfatase · selectin · mass spectrometry · leukocyte adhesion

## INTRODUCTION

Leukocytes roll on selectins under shear flow before adhering firmly to the endothelium and transmigrating to sites of inflammation. Although various candidates have been identified as potential ligands for selectins, P-selectin glycoprotein ligand-1 (PSGL-1) is the only ligand that has conclusively been shown to mediate rolling on P-selectin in vivo [1–3]. P-selectin on endothelium binds PSGL-1 on leukocytes with high affinity and high off-rate [4] to mediate leukocyte rolling during inflammatory cell recruitment. Human PSGL-1, a type I membrane glycoprotein, is a homodimer of 240 kilodaltons with the characteristics of a sialomucin containing numerous clustered S

and T residues [5]. Nascent PSGL-1 has an N-terminal signal peptide (1–18), followed by a propeptide (19–41) that is cleaved by paired, basic amino acid-converting enzymes such that the N-terminal extracellular region of the mature protein begins at residue 42 [6]. There are three clustered Y in a consensus motif for sulfation within the anionic 19 amino acid N-terminal segment that have been shown to be involved in binding to P-selectin. Enzymatic removal of sulfate from Y [7] or metabolic inhibition of Y sulfation [8] and mutational analysis [8, 9] indicate that sulfation of at least one of these tyrosines is required for high affinity binding of P-selectin to PSGL-1. In addition, a specific core 2-based O-glycan at T 57 of the mature protein is required [10]. Synthetic peptides modeled after the N-terminus of human PSGL-1 lacking sulfated Y or core 2-O-glycan do not detectably bind immobilized P-selectin [11]. In a cell-free, bead-rolling assay, the requirement for tyrosine sulfation appears to be relaxed [12]. It is not known how many tyrosines are sulfated in native PSGL-1, but replacement of one or two Y by F in human PSGL-1 shows only subtle differences in the kinetic and mechanical properties of interactions with P-selectin. However, mutation of all three tyrosines severely impairs rolling [13].

Mouse PSGL-1 is similar in size to human PSGL-1 and has a signal peptide, a propeptide, and a single C near the transmembrane domain [14]. Its anionic N-terminus has two rather than three Y, and metabolic inhibition of sulfation abrogates adhesion of myeloid cells to P-selectin-expressing Chinese hamster ovary cells [15]. Epitopes for known function blocking monoclonal antibodies (mAbs) to human PSGL-1, KPL1 [16], and PL1 [17] and mouse PSGL-1, 4RA10 [18], and 2PH1 [2] span the anionic N-terminal segment that is involved in binding to P-selectin.

The objective of the present study was to compare these mAbs for their ability to recognize N-terminal PSGL-1 peptides that are differentially sulfated at the tyrosines. Earlier studies

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had suggested that one of the mAbs to human PSGL-1, KPL1, may require tyrosine sulfation for recognition [16]. We synthesized a series of peptides based on mouse and human PSGL-1 amino acid sequences that differed in the level of sulfation of the N-terminal tyrosine residues. To assess the requirements for antibody binding, we used three different assays: binding of mAbs to immobilized peptides with different levels of sulfation; inhibition of mAbs binding to cells by these peptides; and antibody binding after removing tyrosine sulfate from cells by arylsulfatase. To address a potential influence of charge, we compared the sulfated peptides with phosphorylated peptides.

## MATERIALS AND METHODS

### Materials

All tissue culture media were obtained from Gibco (Gaithersburg, MD). EZ-Link® N-hydroxysuccinimide-biotin was obtained from Pierce (Rockford, IL). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and resins were obtained from Novabiochem (La Jolla, CA). Fmoc-sulfoltyrosine was obtained from Bachem (Torrance, CA). Sulfatase from *Aerobacter aerogens* was obtained from Sigma Chemical Co. (St. Louis, MO).

### Cell lines

The human neutrophilic cell line HL-60 was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The murine hemopoietic progenitor cell line 32Dcl3 was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% FCS and 1% penicillin/streptomycin along with 10% conditioned medium from the murine myelomonocytic cell line WEHI-3B as a source for interleukin-3. WEHI-3B cells were maintained in DMEM containing 10% FCS and 1% penicillin/streptomycin. Culture conditions were at 37°C in 5% CO<sub>2</sub> with twice-a-week passage.

### Antibodies

mAb KPL1, a mouse anti-human PSGL-1 antibody [immunoglobulin G (IgG)<sub>1</sub>] was described previously [16]. mAb PL1 is also a mouse anti-human PSGL-1 (IgG<sub>2</sub>) [19] and was a kind gift of Dr. R. P. McEver (University of Oklahoma Health Sciences Center, Oklahoma City). mAb 4RA10 is a rat anti-mouse PSGL-1 antibody (IgG<sub>2</sub>) raised using recombinant PSGL-1 and recognizes the functional 19 amino acid, N-terminal PSGL-1 [18]. mAb 2PH1 is also a rat anti-mouse PSGL-1 antibody (IgG<sub>2</sub>) and was described previously [2]. The following antibodies were obtained from BD Pharmingen (San Diego, CA): 2PH1, isotype-control mouse IgG<sub>1</sub> κ (clone MOPC-31C), isotype-control rat IgG<sub>1</sub> κ (clone R3-34), goat anti-rat IgG fluorescein isothiocyanate (FITC), rat anti-mouse IgG<sub>2</sub> FITC, and streptavidin FITC. Goat anti-mouse IgG horseradish peroxidase (HRP) and goat anti-rat IgG HRP were obtained from Pierce.

### P-selectin IgM chimeric protein

A plasmid encoding a fusion protein of murine P-selectin and human IgM containing the lectin, epidermal growth factor domain, and the first two consensus repeat domains of mouse P-selectin, fused to the second heavy chain constant region of human IgM, was a kind gift of Dr. J. B. Lowe (University of Michigan Medical School, Ann Arbor). The plasmid DNA was transformed and propagated in *Escherichia coli* MC1061/P3 and was used to transfect COS-7 cells as described [20]. Medium harvested from transfected cells 3 days after the transfection was used for staining cells for flow cytometry experiments.

### Synthesis of peptides

Peptides were synthesized as described [21] except that the cleavage/deprotection time was reduced to 15 min in an effort to minimize tyrosine desulfation. Peptides were purified by reversed-phase high-pressure liquid chromatography (HPLC) and characterized by negative ion mass spectrometry. The

peptides were dissolved in dimethyl sulfoxide:water (1:1) and were assayed for concentration using the bicinchoninic acid protein assay kit (Pierce).

### Mass spectrometry

Peptide solutions were prepared at a concentration of 1 pmol/μL methanol:water:ammonium hydroxide (48:48:4) and analyzed by direct infusion with a TSQ-7000 triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA) equipped with a homebuilt μESI source [22]. The mass spectrometer was operated in the negative-ion mode with a spray voltage of -1.6 kV.

### Enzyme-linked immunosorbant assay (ELISA)

All peptides were diluted in 50 mM carbonate-bicarbonate buffer, pH 9.2, and coated at concentrations starting at 500 ng (for human) and 5 μg (for mouse) per well overnight at 4°C. The coated plates were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 h at room temperature and washed once with 0.05% PBS Tween 20 and incubated with the primary antibodies (KPL1, 1:500 diluted ascites fluid for the sulfated peptides and 5 μg/ml KPL1 for the phosphorylated peptides; PL1, 5 μg/ml; 4RA10, 5 μg/ml; 2PH1, 5 μg/ml; isotype-control mouse IgG<sub>1</sub>, 5 μg/ml; isotype-control rat IgG<sub>1</sub>, 5 μg/ml) for 1 h at room temperature. The plates were then washed three times and incubated with the secondary antibody [goat anti-mouse HRP (Pierce) for KPL1, PL1, and mouse isotype control; goat anti-rat HRP (Pierce) for 4RA10, 2PH1, and rat isotype control] at a dilution of 1:4000 for 1 h at room temperature and were then washed again three times and detected with orthophenyldiamine tetrahydrochloride and hydrogen peroxide. Optical density (OD) was measured at 450 nm using a Labsystems plate reader (Thermo Labsystems, Helsinki, Finland).

### Blocking of mAbs with peptides

Each anti-mouse and anti-human mAb (1 μg) was preincubated with 10 μg of each of the corresponding peptide for one-half hour on ice in PBS containing 1% BSA (fluorescein-activated cell sorter (FACS) buffer). For flow cytometry, HL-60 and 32Dcl3 cells were washed twice at 100 g for 5 min with cold FACS buffer, were resuspended in KPL1 or PL1 (10 μg/ml), mouse IgG1 isotype (10 μg/ml), mAb preincubated with peptide (for HL-60), 4RA10 or 2PH1 (10 μg/ml), rat IgG1 isotype (10 μg/ml), or mAb preincubated with peptide (for 32Dcl3), and were incubated on ice for 30 min. Cells were then washed twice with cold FACS buffer and incubated with the secondary antibody (anti-mouse IgG FITC for KPL1 and PL1 and anti-rat IgG FITC for 4RA10 and 2PH1, both at 5 μg/ml) for 30 min on ice. After two washes, the samples were analyzed by flow cytometry in FACS Calibur (BD Biosciences, Franklin Lakes, NJ) using Cellquest software.

### Competition between mAbs for cell surface PSGL-1

KPL1 and PL1 were biotinylated using EZ-Link NHS-LC-Biotin. Biotinylated KPL1 (5 μg/ml) and increasing concentrations of unlabeled KPL1 or PL1 were coincubated with  $1 \times 10^6$  HL-60 cells on ice for 30 min. After two washes with FACS buffer, cells were incubated with streptavidin FITC at 5 μg/ml on ice for 30 min and were washed twice with FACS buffer and analyzed by flow cytometry. In a reverse experiment, 5 μg/ml biotinylated PL1 was coincubated with unlabeled PL1 or KPL1. Similar experiments were conducted in 32Dcl3 cells using biotinylated 2PH1 and biotinylated 4RA10 (biotinylated using EZ-Link NHS-LC-Biotin).

### Arylsulfatase digestion

HL-60 cells were grown in RPMI-1640 medium containing 10% FCS and 1% penicillin/streptomycin. Cells (1 ml containing  $10^6$ ) were added per well in a 24-well tissue culture plate, and the cells were treated with not any, 0.2 U, 1 U, or 2 U arylsulfatase overnight at 37°C. The cells were then washed twice with FACS buffer, and for each treatment, they were stained with KPL1 and P-selectin IgM chimera separately. Goat anti-mouse IgG HRP was used to detect KPL1, and biotinylated goat anti-human IgM (5 μg/ml) followed by streptavidin FITC (5 μg/ml) was used to detect P-selectin IgM binding.

## RESULTS

### Differentially modified N-terminal PSGL-1 peptides

Three sets of PSGL-1 peptides based on the N-terminal sequence of the human protein (Fig. 1A) and one set of peptides based on the N-terminal sequence of the mouse protein (Fig. 1B) were synthesized using Fmoc-protected amino acids, purified by HPLC and analyzed for sequence identity by their mass spectrometry (MS)/MS spectra. The shorter, 11-mer human peptides with 0, 1, 2, or 3 sulfated tyrosines were designated as 0s, 1s (at residue 46), 2s (at residues 46 and 48), and 3s (at residues 46, 48, and 51), and the corresponding 17-mer human peptides were designated as 0S, 1S (at residue 46), 2S (at residues 46 and 48), and 3S (at residues

<b>A</b>	
Human PSGL-1	42 Q-A-T-E-Y-E-Y-L-D-Y-D-F-L-P-E-T-E-P-E-M-57
	46          48          51
	SO <sub>2</sub> SO <sub>2</sub> SO <sub>2</sub>
Synthetic Peptides	
0s	Q-A-T-E-Y-E-Y-L-D-Y-D
0S	Q-A-T-E-Y-E-Y-L-D-Y-D-F-L-P-E-T-E
1s	Q-A-T-E-Y-E-Y-L-D-Y-D
	46
	SO <sub>2</sub>
1S	Q-A-T-E-Y-E-Y-L-D-Y-D-F-L-P-E-T-E
	46          48
	SO <sub>2</sub> SO <sub>2</sub>
2s	Q-A-T-E-Y-E-Y-L-D-Y-D
	46          48
	SO <sub>2</sub> SO <sub>2</sub>
2S	Q-A-T-E-Y-E-Y-L-D-Y-D-F-L-P-E-T-E
	46          48          51
	SO <sub>2</sub> SO <sub>2</sub> SO <sub>2</sub>
3s	Q-A-T-E-Y-E-Y-L-D-Y-D
	46          48          51
	SO <sub>2</sub> SO <sub>2</sub> SO <sub>2</sub>
3S	Q-A-T-E-Y-E-Y-L-D-Y-D-F-L-P-E-T-E
	46          48          51
	SO <sub>2</sub> SO <sub>2</sub> SO <sub>2</sub>
SP1	A-Y-Q-P-E-Y-T-L-E-F-E-Y-D-L-T-D-E
SP2	E-P-A-D-T-E-Y-Q-L-E-T-Y-F-L-D-E-Y
<b>B</b>	
Mouse PSGL-1	42 Q-V-V-G-D-D-D-F-E-D-P-D-Y-T-Y-N-T-D-P-P-E-58
	54          55          56          58
	SO <sub>2</sub> SO <sub>2</sub> SO <sub>2</sub> SO <sub>2</sub>
Synthetic Peptides	
0s	Q-V-V-G-D-D-D-F-E-D-P-D-Y-T-Y-N-T
1s	Q-V-V-G-D-D-D-F-E-D-P-D-Y-T-Y-N-T
	54
	SO <sub>2</sub>
2s	Q-V-V-G-D-D-D-F-E-D-P-D-Y-T-Y-N-T
	54          55
	SO <sub>2</sub> SO <sub>2</sub>

Fig. 1. Amino acid sequences of nonsulfated and differentially sulfated human and mouse PSGL-1 peptides. Human and mouse peptides were synthesized using Fmoc-protected amino acids based on the N-terminal amino acid sequences of human and mouse PSGL-1, respectively. (A) Human and (B) mouse synthetic PSGL-1 N-terminal peptides with differential tyrosine sulfations at the indicated residues are shown. SP1 and SP2 represent sequences of scrambled peptides that were used as negative controls. Human PSGL-1 peptides (17-mers) were also synthesized with tyrosine phosphates at the indicated residues.

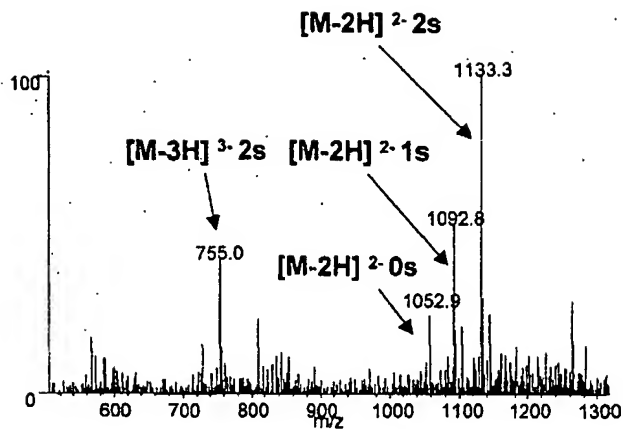


Fig. 2. A representative MS spectrum for synthetic sulfated peptides. HPLC-purified, differentially sulfated peptides were characterized by negative ion mass spectrometry. An MS spectrum for mouse 2S peptide shows relative abundance as a function of mass-to-charge ratio ( $m/z$ ) containing the predominant 2S peak (1133.3) with contributions from 1S (1092.8) and 0S (1052.9) peptides. The 2S species is represented by a combination of the doubly deprotonated  $[M-2H]^{2-}2S$  and the triply deprotonated  $[M-3H]^{3-}2S$  forms, which make up for 60% of the mixture, and the other 40% comprise the desulfated species.

46, 48, and 51) for the sulfated and as 0P, 1P (at residue 46), 2P (at residues 46 and 48), and 3P (at residues 46, 48, and 51) for the phosphorylated tyrosines. The 17-mer mouse peptides were similarly called 0S, 1S, and 2S. Two scrambled peptides, SP1 and SP2, were also prepared. Nonsulfated peptides were >90% pure as determined by MS. Preparations of sulfated peptides contained significant amounts of desulfated peptides. All of the phosphorylated peptides were >95% pure as determined by MS. A representative MS spectrum for the mouse peptide 2S shows the presence of 1S and 0S species, although they are less abundant than the 2S species (Fig. 2).

### Antibody binding to plate-bound peptides

In an ELISA, KPL1 and PL1 showed dose-dependent binding to the 17-mer human peptides 0S, 1S, 2S, and 3S (Fig. 3, A and B). All peptides, irrespective of the degree of tyrosine sulfation, showed antibody binding, which increased with increasing peptide concentration. The 11-mer human peptides 0s, 1s, 2s, and 3s bound neither PL1 nor KPL1. Neither the 11-mer nor the 17-mer peptides bound the isotype-matched control antibody (mouse IgG<sub>1</sub>). The differentially phosphorylated 17-mer human peptides showed dose-dependent antibody binding, and the specificity of binding was confirmed using scrambled peptides SP1 and SP2, neither of which bound the antibodies (data not shown; and Fig. 3, A and B). The mouse peptides 0S, 1S, and 2S showed dose-dependent binding to 4RA10 and 2PH1 (Fig. 3, C and D), and the binding was specific as shown by lack of antibody binding to scrambled peptides SP1 or SP2 (Fig. 3, C and D). Also, the mouse peptides did not bind the isotype-matched control antibody (rat IgG<sub>1</sub>).

### Blocking of PSGL-1 mAbs binding by peptides

Preincubating anti-PSGL-1 mAbs with peptides decreased subsequent binding of the antibodies to native PSGL-1 on

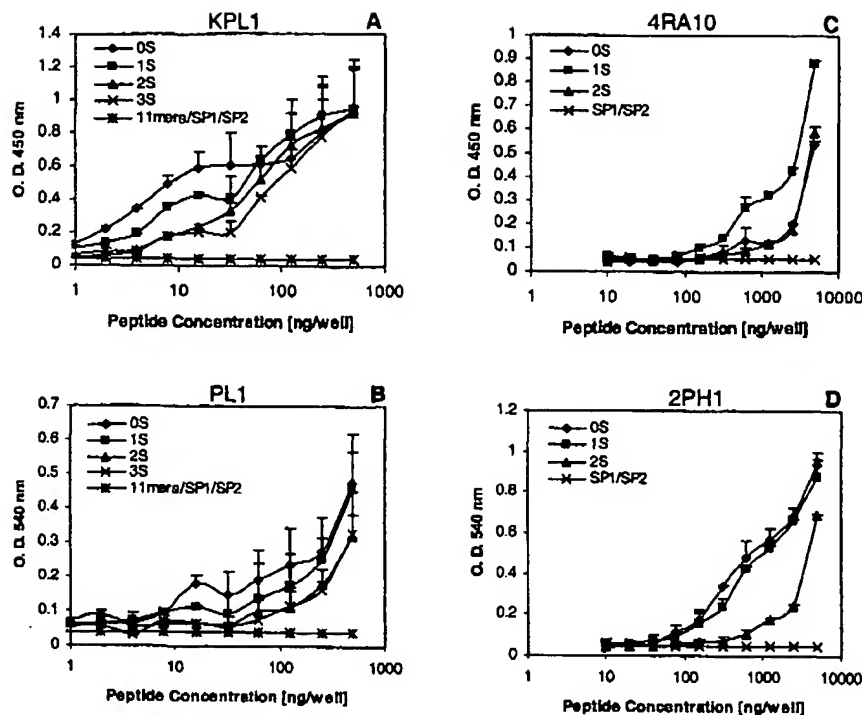


Fig. 3. mAbs bind N-terminal PSGL-1 peptides in an ELISA, irrespective of the degree of tyrosine sulfation. Human or mouse peptides were coated on an ELISA plate in duplicate wells and incubated with anti-human mAb, KPL1 or PL1, or anti-mouse mAb, 4RA10 or 2PH1, respectively. HRP-conjugated, secondary antibodies were used to detect primary antibody binding. Anti-human PSGL-1 mAb KPL1 (A) and PL1 (B) show dose-dependent binding to the 17-mer peptides (0S, 1S, 2S, or 3S) but not to scrambled control peptides SP1 or SP2 or 11-mer peptides with or without sulfation (0s, 1s, 2s, or 3s). Anti-mouse PSGL-1 mAb 4RA10 (C) and 2PH1 (D) show dose-dependent binding to 0S, 1S, or 2S mouse peptides. The antibodies do not bind scrambled peptides SP1 or SP2. Isotype-control antibodies, mouse IgG<sub>1</sub> (for anti-human mAb KPL1 and PL1) and rat IgG<sub>1</sub> (for anti-mouse mAb 4RA10 and 2PH1), were tested on human and mouse peptides, respectively, but did not show any binding. Data are represented as mean + SD for  $n = 3$ .

cells. KPL1 staining on HL-60 cells was significantly reduced when the antibody was preincubated with 1S, 2S, and 3S peptides (Fig. 4A). The 0S peptide could also displace KPL1 binding though not as well as the other three. The 11-mer human peptides 0s, 1s, 2s, and 3s did not inhibit KPL1 binding to HL-60 cells (Fig. 4B). Although PL1 did not detectably bind 11-mer peptides by ELISA, PL1 binding to HL-60 cells was inhibited by the 17-mers (Fig. 4C) and the 11-mers (Fig. 4D).

With both sets of peptides, the sulfated peptides inhibited better than the nonsulfated peptides. To test if the differences in the level of inhibition seen with the sulfated peptides on KPL1 binding was due to the charge on the sulfate moieties, we used similar charge-bearing, tyrosine-phosphorylated peptides for inhibition of KPL1 binding. As shown in Figure 5, even at concentrations spanning two logs (A, 0.1  $\mu\text{g}/\mu\text{g}$  KPL1; B, 1  $\mu\text{g}/\mu\text{g}$  KPL1; C, 10  $\mu\text{g}/\mu\text{g}$  KPL1), there were no significant

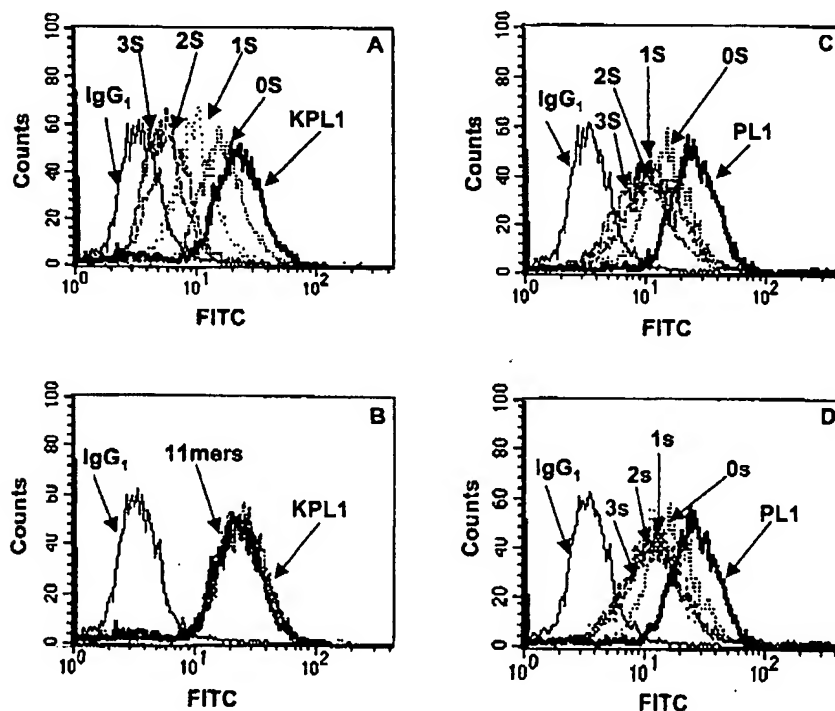


Fig. 4. Differentially sulfated peptides compete for antibody binding to native human PSGL-1. Each antibody (1  $\mu\text{g}$ ) was preincubated with 10  $\mu\text{g}$  each peptide for 30 min at 4°C before being added to  $1 \times 10^6$  HL-60 cells. Antibody binding to HL-60 cells was detected using a FITC-labeled secondary antibody and analyzed by flow cytometry. KPL1 binding to HL-60 cells is decreased in the presence of 17-mer peptides 0S, 1S, 2S, or 3S (A) but not by the 11-mer peptides 0s, 1s, 2s, or 3s (B). PL1 binding to HL-60 cells is decreased by 11-mer (0s, 1s, 2s, or 3s) and 17-mer peptides (0S, 1S, 2S, or 3S; D and C, respectively). IgG<sub>1</sub>, Mouse isotype-control antibody binding. Data are representative of four independent experiments.

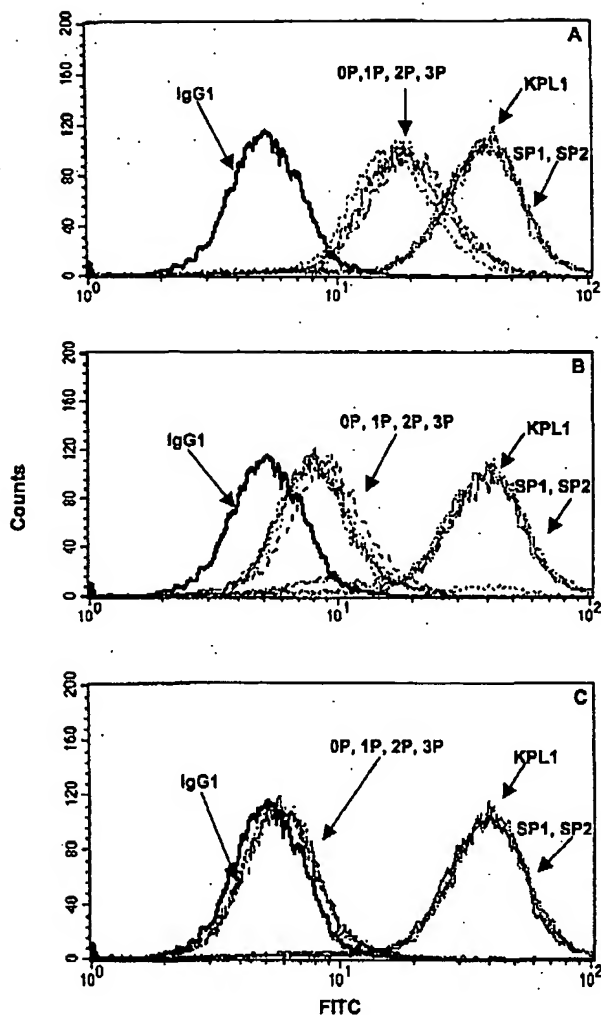


Fig. 5. Differentially phosphorylated peptides compete for KPL1 binding to HL-60 cells. KPL1 (1  $\mu$ g) was preincubated with varying concentrations of differentially phosphorylated peptides OP, 1P, 2P, or 3P at 4°C for 30 min before being added to  $1 \times 10^6$  HL-60 cells. Antibody binding to HL-60 cells in the presence and absence of peptides was detected using a FITC-labeled secondary antibody and was analyzed by flow cytometry. KPL1 binding to HL-60 cells can be reduced by OP, 1P, 2P, or 3P peptide at concentrations of 0.1  $\mu$ g (A), 1  $\mu$ g (B), and 10  $\mu$ g (C) per  $\mu$ g KPL1 in a dose-dependent manner. The scrambled peptides SP1 and SP2 do not affect KPL1 binding at any concentration. IgG<sub>1</sub>, Mouse isotype-control antibody binding. Result shown is representative of three experiments conducted.

differences in binding inhibition observed with the differentially phosphorylated peptides. The mouse peptides OS, 1S, and 2S completely inhibited 2PH1 staining on 32Dcl3 cells irrespective of the degree of tyrosine sulfation (Fig. 6A). 4RA10 staining on 32Dcl3 cells was also blocked by all three peptides, although not as completely as in the case of 2PH1 (Fig. 6B).

#### KPL1 competes for PL1 binding on HL-60 cells

When a fixed concentration of biotinylated PL1 and increasing concentrations of unlabeled KPL1 or PL1 were coincubated with HL-60 cells, PL1 staining was reduced as a function of KPL1 or PL1 concentration (Fig. 7A). However, KPL1 stain-

ing on HL-60 cells, which was reduced as a function of unlabeled KPL1, could not be blocked by even tenfold higher concentrations of PL1 (Fig. 7B). This suggests that KPL1 might have a higher affinity than PL1 for native PSGL-1 on HL-60 cells. In addition, 4RA10 and 2PH1 did not compete for PSGL-1 on 32Dcl3 cells. Although staining of biotinylated 4RA10 and biotinylated 2PH1 was reduced as a function of increasing concentrations of unlabeled 4RA10 and unlabeled 2PH1, respectively (Fig. 7, C and D), there was no reduction in staining of one while coincubated with increasing concentrations of the other, suggesting that their epitopes do not overlap (Fig. 7, C and D).

#### Recognition of native human PSGL-1 by KPL-1 is independent of tyrosine sulfation

HL-60 cells were treated with the enzyme arylsulfatase, which cleaves sulfate from tyrosine. KPL1 staining of native PSGL-1 on HL-60 cells was not significantly altered after arylsulfatase treatment (Fig. 8). As a positive control, P-selectin IgM staining of arylsulfatase-treated cells decreased as a function of the concentration of the enzyme used for removing sulfate. It is well known that P-selectin binding to PSGL-1 requires sulfation of at least one of the three N-terminal tyrosines [8]. Reduction in P-selectin IgM staining indicates successful removal of sulfates from PSGL-1, but this did not affect KPL1 staining. Therefore, KPL1 recognition of PSGL-1 does not require tyrosine sulfation.

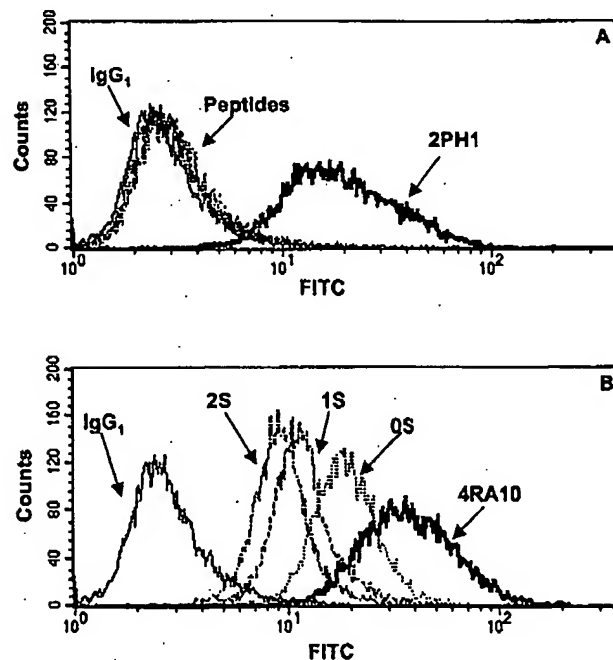


Fig. 6. Peptides compete for antibody binding to native, murine PSGL-1. 2PH1 or 4RA10 (1  $\mu$ g) was preincubated with 10  $\mu$ g each OS, 1S, and 2S for 30 min at 4°C before being incubated with  $1 \times 10^6$  32Dcl3 cells. Antibody binding to 32Dcl3 cells in the presence and absence of peptides was detected using a FITC-labeled secondary antibody and was analyzed by flow cytometry. In the presence of OS, 1S, or 2S mouse peptides 2PH1 (A) and 4RA10 (B), binding to native PSGL-1 on 32Dcl3 cells is reduced. Control, Rat IgG<sub>1</sub> isotype-control antibody binding. Data shown are representative of two independent experiments.



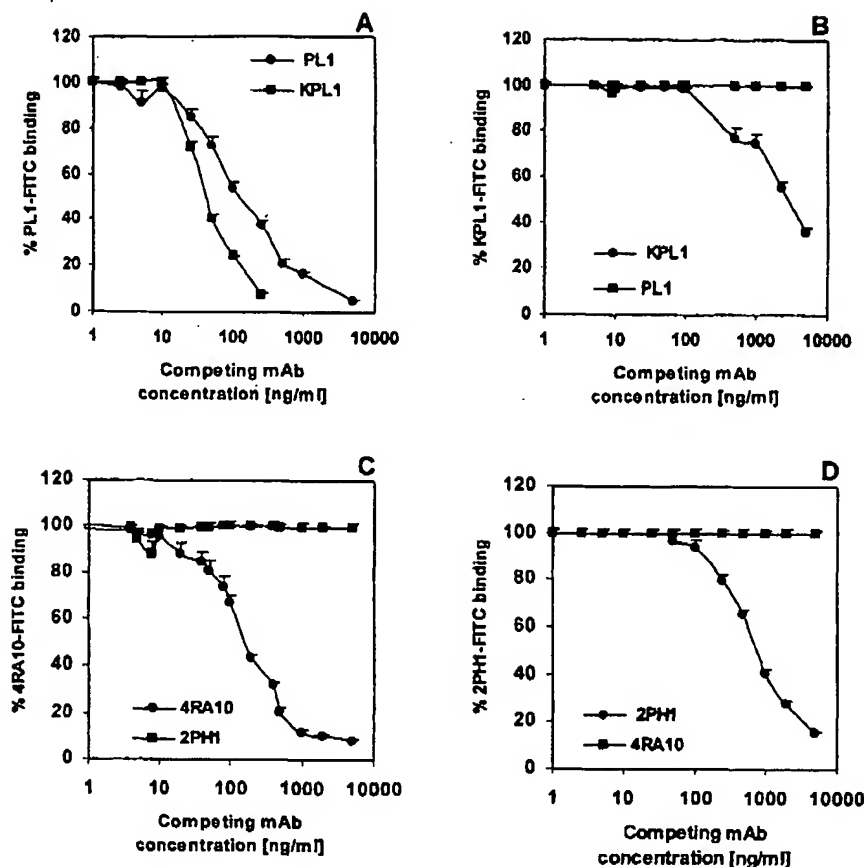


Fig. 7. Competition between mAb for cell surface PSGL-1. For the competition assay, cells were co-incubated with a fixed concentration of one antibody (FITC-labeled) and varying concentrations of the competing antibody (unlabeled). The effect of competing antibody on the binding of the primary antibody was analyzed by flow cytometry. (A) % PL1-FITC binding to HL-60 cells as a function of increasing concentrations of PL1 (●) or KPL1 (■). (B) % KPL1-FITC binding to HL-60 cells as a function of increasing concentrations of KPL1 (●) or PL1 (■). (C) % 4RA10 binding to 32Dcl3 cells as a function of increasing concentrations of 4RA10 (●) or 2PH1 (■). (D) % 2PH1 binding to 32Dcl3 cells as a function of increasing concentrations of 2PH1 (●) or 4RA10 (■). Mean fluorescence intensity observed in the absence of the competing antibody is expressed as 100%. Data are represented as mean  $\pm$  SD for  $n = 3$ .

## DISCUSSION

The present study analyzes the binding specificities of two mAbs to human PSGL-1 and two mAbs to mouse PSGL-1 in relation to the degree of tyrosine sulfation at the N-terminus. PSGL-1-mediated, high affinity interaction with P-selectin can be inhibited by each of these mAbs. mAbs KPL1 [16] and PL1 [19] recognize epitopes in the functionally important N-terminus of human PSGL-1, and mAbs 2PH1 [2] and 4RA10 [18] recognize functional epitopes in the N-terminus of mouse PSGL-1.

All mAbs used in this study block binding of PSGL-1 to P-selectin, suggesting that the antibody epitopes span the P-selectin-binding site [23]. PL1, which was raised against purified PSGL-1 from human neutrophils [19], is known to recognize peptides spanning residues 49–62 of mature PSGL-1 including the LPETE motif [17]. Conversely, KPL1 was raised against recombinant PSGL-1 and interacts with the tyrosine sulfation consensus motif of PSGL-1 comprising residues YEYLDYD [46–52] at the N-terminus [16]. In the present study, PL1 and KPL1 bound immobilized, 17-mer, N-terminal human PSGL-1 peptides irrespective of the degree of tyrosine sulfation. This suggests that recognition of PSGL-1 by either of them is sulfation-independent. This is further supported by equal binding of peptides with three, two, one, or no tyrosine phosphates. The shorter 11-mers did not show any reactivity above background with PL1 or KPL1. Absence of PL1 binding to the 11-mers agrees with earlier reports on PL1

binding to fusion proteins containing fragments of the extracellular domain of PSGL-1, which suggested that PL1 optimally binds to a 14-residue epitope that spans amino acids 49–62, although the antibody can recognize shorter, 8-residue peptides with the LPETE motif not contained in the 11-mer [17]. Absence of reactivity of KPL1 to the 11-mer containing the YEYLDYD sequence suggests that the KPL1 epitope extends beyond that sequence.

To address the possibility that the adsorption of 11-mer peptides to the plastic ELISA plate may prevent availability of the KPL1 epitope, we conducted a soluble-phase flow cytometry assay. The 11-mer peptides were able to compete for PL1 but not KPL1 binding to HL-60 cells. The difference in the behavior of 11-mer peptides with PL1 between the solid phase and the fluid phase assays might be a result of the reported fact that amino acid residues, which appear to play no role when tested by ELISA, influence the kinetics of binding under dynamic conditions by stabilizing the conformation [24]; thus, the peptides would be able to assume the necessary secondary structures for antibody binding that were not permitted in the solid phase. KPL1 binding to HL-60 cells was blocked by 17-mers but not 11-mers. This assay suggests that the KPL1 epitope extends beyond the aspartic acid residue at position 52 of mature PSGL-1.

All the 17-mers inhibit KPL1 and PL1 binding to HL-60 cells. It is possible that initial binding of the 17-mer peptides to either mAb causes a change in the antibody conformation, thereby inhibiting antibody binding to PSGL-1 on HL-60 cells.

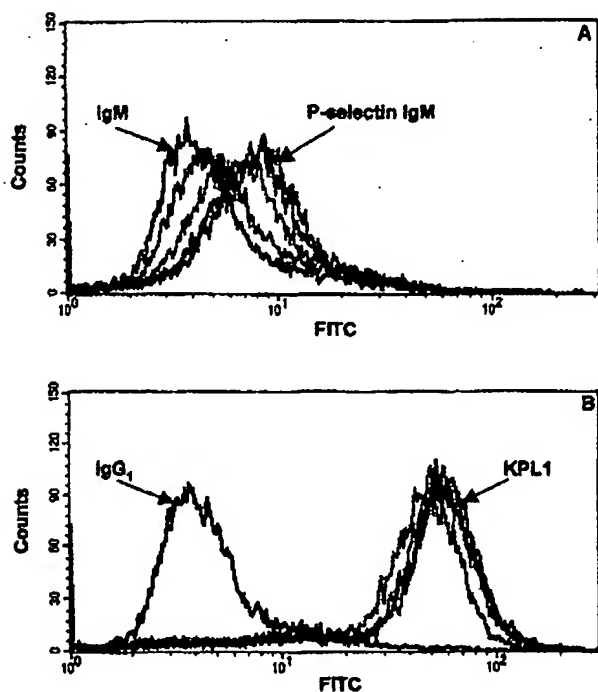


Fig. 8. KPL1 binding does not require tyrosine sulfation. HL-60 cells were treated overnight with increasing concentrations of arylsulfatase (0.2 U, 1 U, or 2 U/10<sup>6</sup> cells/ml) to remove sulfate groups from tyrosine residues. Untreated and arylsulfatase-treated HL-60 cells were stained with KPL1 and P-selectin IgM and analyzed by flow cytometry. Binding of P-selectin IgM (A, IgM: anti-human IgM binding) and KPL1 (B, IgG<sub>1</sub>: mouse IgG<sub>1</sub> isotype-control binding) to HL-60 cells in the absence and presence of treatment with increasing concentrations (0.2 U, red; 1 U, green; and 2 U, brown) of arylsulfatase is shown. Data are representative of two independent experiments.

Similar conformational changes have been reported for class II major histocompatibility complex proteins after peptide binding [25]. For KPL1, unlike for PL1, the triply sulfated peptide seems to inhibit better than doubly, singly, or the nonsulfated peptide. Interestingly, phosphorylated peptides did not show this behavior but blocked KPL1 binding equally well, irrespective of phosphorylation. The scrambled peptides had no effect at any concentration. Kinetic measurements of antibody binding to peptides of the coat protein of tobacco mosaic virus have shown that even conservative changes within the epitopes resulted in a significantly higher dissociation rate of the antibodies tested [24]. Based on this, we speculate that sulfated peptides may dissociate more slowly or associate faster with KPL1. As a result, the triply sulfated peptide might perturb KPL1 binding to HL-60 cells more efficiently. Such a kinetic effect of tyrosine sulfation would not be expected in an antibody binding assay to immobilized peptide, which represents more a test of equilibrium affinity. Differentially phosphorylated peptides inhibit KPL1 binding to the same degree as opposed to differentially sulfated peptides, suggesting that the size of the substituted group on tyrosine but not its charge determines the extent to which antibody binding is perturbed. Although there are no reports in the literature that present evidence for kinetic differences in binding of antibodies to differentially sulfated or phosphorylated proteins or peptides, it

has been shown that sulfation of synthetic peptides within the  $\gamma$  chain of human fibrin increases the competition for binding of native human fibrin to thrombin [26].

KPL1 failed to recognize a PSGL-1 mutant in which all three tyrosines were replaced by phenylalanines [16]. Based on this finding, it was concluded that KPL1 required sulfated tyrosine residues for recognition. However, our data show that nonsulfated tyrosine residues at positions 46, 48, and 52 are sufficient for recognition, suggesting that the substitution of F for Y rather than the absence of sulfate abolished KPL1 binding to the mutant PSGL-1.

To investigate whether KPL1 binding to native cellular PSGL-1 requires sulfation, we treated HL-60 cells with a bacterial arylsulfatase, which cleaves sulfates from tyrosine residues within proteins but does not digest sulfated carbohydrates [7]. We compared KPL1 binding with P-selectin IgM fusion protein binding. P-selectin IgM fusion protein is known to require tyrosine sulfation for binding to PSGL-1 [20]. Our data show that removal of sulfate from tyrosines significantly decreases P-selectin-IgM binding but not KPL1 binding. In a previous study, arylsulfatase treatment of whole cell lysates was shown to abrogate KPL1 binding to PSGL-1 in a Western blot [16]. However, it was not determined whether binding of P-selectin (which requires sulfation of tyrosines at the N-terminus of PSGL-1) or PL1 (which recognizes an overlapping N-terminal epitope) was affected by the treatment. Binding of PL2 (another PSGL-1 mAb) was unchanged after arylsulfatase treatment, but the PL2 epitope maps far away from the N-terminus [17]. It is therefore possible that the sulfatase treatment or a protease contamination affected the KPL1 epitope.

As KPL1 and PL1 block PSGL-1 function by binding to overlapping epitopes within the anionic N-terminus, we compared their binding to PSGL-1 for possible affinity differences. We tested the ability of one antibody to displace binding of the other, using HL-60 promyelocytes. KPL1 cross-competes with PL1 and displaces PL1 binding in a dose-dependent manner. PL1, on the other hand, does not inhibit KPL1 binding. These findings suggest that the epitopes for KPL1 and PL1 are at least partially overlapping, and KPL1 may have a higher affinity for PSGL-1 than PL-1. However, alternative explanations exist; for example, KPL1 may change PSGL-1 conformation in such a way that PL1 can no longer bind without physically overlapping the PL-1 epitope.

Murine PSGL-1 is structurally similar to human PSGL-1 and has an anionic N-terminal sequence with two rather than three tyrosines [5]. mAb 2PH1 was raised against an N-terminal mouse PSGL-1 peptide [2], while mAb 4RA10 was raised using recombinant PSGL-1 [18], and both block PSGL-1 function in the mouse [2, 18]. We find that 2PH1 and 4RA10 bind to the N-terminal 17 amino acids of murine PSGL-1, independent of tyrosine sulfation. Peptides with and without sulfated tyrosines are also able to compete for antibody binding to native PSGL-1 on 32Dcl3 cells. Although both mAbs recognize the N-terminus of murine PSGL-1 and both block PSGL-1 function, they seem to bind to nonoverlapping epitopes because they are unable to compete for each other on 32Dcl3 cells.

Taken together, our studies show that function-blocking antibodies to human or mouse PSGL-1 recognize N-terminal

peptides independent of tyrosine sulfation. We conclude that, in contrast to P-selectin binding, mAb binding to human and mouse PSGL-1 does not require tyrosine sulfation.

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